A QUANTITATIVE PROTEOMICS STUDY TO INVESTIGATE KEY PROTEINS INVOLVED IN OXIDATIVE PATHWAYS IN PATIENTS WITH ALZHEIMER'S DISEASE

by
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ABSTRACT

Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative disease that is characterized by accumulation of amyloid beta forming senile plaques and the deposition of microtubule-associated tau proteins into neurofibrillary tangles. It is primarily a disease of synaptic dysfunction and the loss of synapses can be correlated with cognitive decline. Oxidative stress also has been hypothesized to play an increasingly important role in pathogenesis of late onset AD. Using brain samples from participants in the Baltimore Longitudinal Study of Aging (BLSA) we developed and optimized a protein extraction protocol to investigate key proteins involved in oxidative pathways and the temporal relationship of synaptic dysfunction to onset of cognitive dysfunction. Using age matched samples from the BLSA who are cognitively normal, have mild cognitive impairment and those with dementia we investigated the role of TOMM40, COV-IV, synaptophysin and PSD95. Samples from the superior and middle temporal gyrus (SMTG) and middle frontal gyrus (MFG) were homogenized and soluble extracellular, cellular and insoluble fractions were extracted. Using semi-quantitative Western blotting the concentration of these proteins were measured and correlated with the onset of clinical symptoms of Alzheimer's disease.

Readers: Dr. Abhay Moghekar, PhD; Dr. Kevin Yarema, PhD; Dr. Srinivasan Chandrasegaran, PhD

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INTRODUCTION

Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative disease and the most common cause of dementia. In Alzheimer's disease, neuronal damage eventually affects parts of the brain that enable a person to carry out basic bodily functions such as walking, swallowing etc. More than 35 million people worldwide have AD - a deterioration of memory and other cognitive domains that leads to death within 3 – 9 years after diagnosis. Dementia is also caused by other diseases and is characterized by a decline in memory, problem-solving, language and other cognitive skills that affects a person's ability to perform everyday activities [1].

Alzheimer's disease symptoms vary among individuals. The most common initial symptom is a gradually worsening ability to remember new information. The first neurons to malfunction and die are usually neurons in brain regions involved in forming new memories [2]. The common symptoms of AD are as follows:

- Memory loss that disrupts daily life.
- Difficulty completing familiar tasks at home or work
- Trouble understanding visual images and spatial relationships.
- Misplacing things and losing the ability to retrace steps.
- Decreased or poor judgment.
- Challenges in planning or solving problems.
- Confusion with time or place.
- Withdrawal from work or social activities.

- Changes in mood and personality, including apathy and depression.

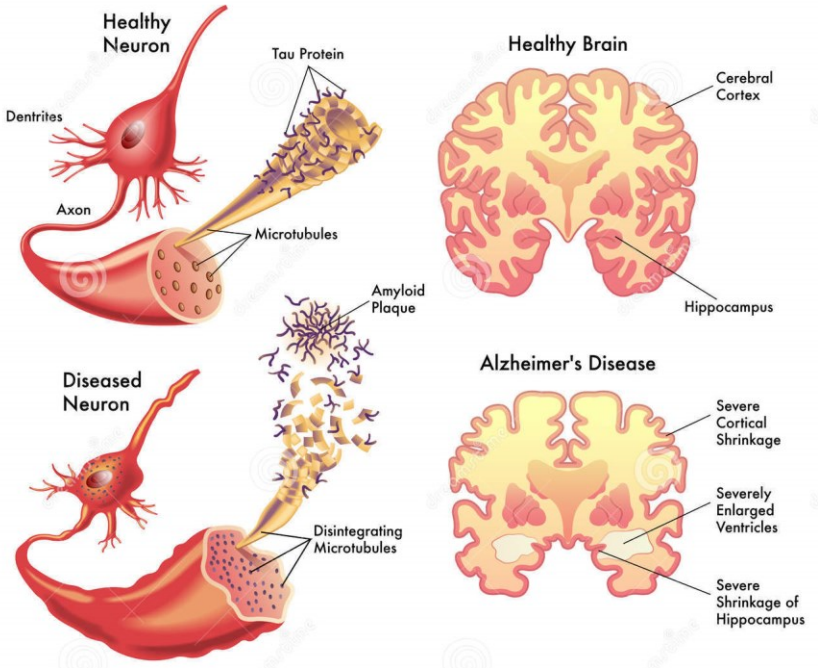

Figure 1: Normal brain vs an Alzheimer's disease brain showing shrinkage of hippocampus and cerebral cortex; and enlarged ventricles

(Source: Courtesy of ADEAR - Alzheimer's disease Education and Referral Center, a service of the National Institute on Aging)

The pace at which symptoms advance from mild to moderate to severe varies from person to person. The principal risk factor for AD is age. The incidence of the disease doubles every 5 years after 65 years of age. As the disease progresses, cognitive and functional abilities decline. People need help with basic activities of daily living. They lose their ability to communicate; fail to recognize loved ones and become reliant on around-the-clock care. [3]

PROTEIN ABNORMALITIES IN ALZHEIMER'S DISEASE:

For over 100 years, scientists have recognized a strong correlation between the clinical signs of late-life dementia and the presence in brain of abnormal protein deposits. The brains of people with advanced AD show dramatic shrinkage from cell loss and widespread debris from dead and dying neurons. Accumulation of misfolded proteins in the ageing brain results in oxidative and inflammatory damage, which in turn leads to energy failure and synaptic dysfunction In AD, these deposits contain aggregated peptide fragments of various proteins, including the amyloid precursor protein (APP), the microtubule-associated tau protein and others. The accumulation of beta-amyloid forming senile plaques outside neurons and the deposition of microtubule-associated tau proteins forming neurofibrillary tangles inside neurons are two of several brain changes believed to contribute to the development of AD. The accumulation of amyloid beta interferes with the neuronal communication at synapses and contributes to cell death. Tau neurofibrillary tangles block the transport of nutrients and other essential molecules inside neurons. Subcortical structures are lost including 75% or more of the cells of the basal nucleus of Meynert, the dorsal raphe and the locus coeruleus [4].

The important pathological features of Alzheimer's disease are cerebral plaques laden with β-amyloid peptide (Aβ) and neurofibrillary tangles in medial temporal-lobe structures [5]. Aβ peptides are natural products of metabolism consisting of 36 to 43 amino acids. Beta amyloid peptides originate from proteolysis of the amyloid precursor protein by the sequential enzymatic actions of beta-site amyloid precursor protein–

cleaving enzyme 1 (BACE-1), a β-secretase, a γ-secretase, a protein complex with presenilin 1 at its catalytic core. An imbalance between production, clearance and aggregation of peptides causes Aβ to accumulate, and this excess may be the initiating factor in Alzheimer's disease. This idea called the "amyloid hypothesis," is based on studies of genetic forms of Alzheimer's disease, including Down's syndrome and evidence that $A\beta_{42}$ is toxic to cells [1].

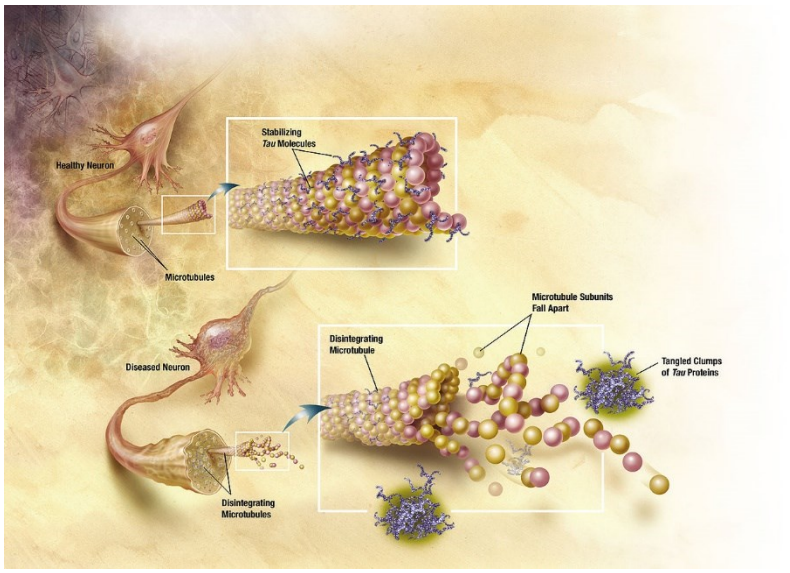

Figure 2: Changes in tau protein leading to disintegration of microtubules in brain cells

(Source: Courtesy of ADEAR - Alzheimer's disease Education and Referral Center, a service of the National Institute on Aging)

Neurofibrillary tangles are seen in Alzheimer's disease and other neurodegenerative disorders called tauopathies. Tau promotes assembly and stability of microtubules and vesicle transport. The number of neurofibrillary tangles is a pathologic marker of the severity of Alzheimer's disease. An abnormally hyperphosphorylated and aggregated

form of tau is known to be a component of tangles. Hyperphosphorylated tau is insoluble, lacks affinity for microtubules and self-associates into helical filament structures. [1]

MILD COGNITIVE IMPAIRMENT:

Mild Cognitive Impairment (MCI) is an intermediate stage between the expected cognitive decline of normal aging and the more pronounced decline of dementia. It involves problems with memory, language, thinking, and judgment that are greater than typical age-related changes. These changes associated with MCI aren't severe enough to interfere with day-to-day life and ordinary activities. Unlike dementia, where cognitive abilities gradually decline, the memory deficits in MCI may remain stable for years. However it is seen that some individuals with MCI will develop Alzheimer's disease. The ability to learn and retain new information is called episodic memory. An impairment in this kind of memory is most commonly seen in MCI patients who subsequently progress to a diagnosis of AD.

- MCI that primarily affects memory is known as amnestic MCI wherein a person may start to forget important information that he or she would previously have recalled easily.
- MCI that affects thinking skills other than memory is known as non-amnestic MCI. Thinking skills are affected including the ability to make sound decisions and judging the time or sequence of steps needed to complete a complex task [6].

Studies have shown that there are a variety of episodic memory tests that are useful for identifying those MCI patients who have a high likelihood of progressing to AD dementia within a few years whereby it is possible to determine retention over a delay. It

is important to examine domains in addition to memory such as executive functions (reasoning, problem-solving), language (fluency, speech, and comprehension), visuospatial skills, and attentional control. Many validated clinical neuropsychological measures are available to assess these cognitive domains, including the Trail Making Test (executive function), the Boston Naming Test, letter and category fluency (language), figure copying (spatial skills) and digit span forward (attention) [7].

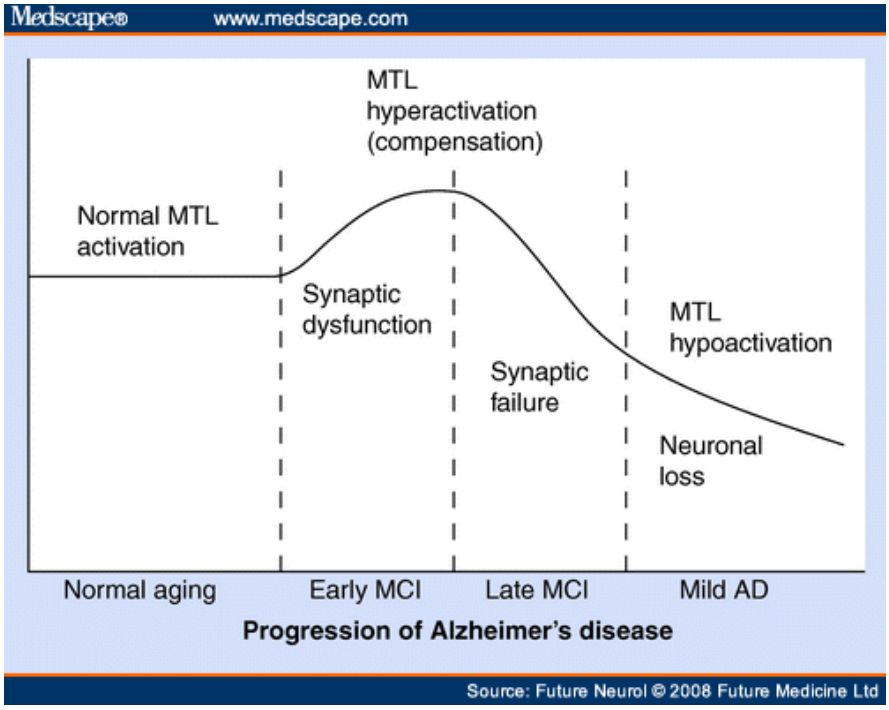

Figure 3: Progression of Alzheimer's disease

(Source: Pihlajamäki, Maija, and Reisa A. Sperling. "fMRI: use in early Alzheimer's disease and in clinical trials." (2008): 409-421)

There are genetic influences on the development of late onset AD. The presence of an ε4 allele in the APOE gene is the only gene broadly accepted as increasing risk for late-onset AD, while the ε2 allele decreases risk. Evidence suggests that an individual who

meets the clinical, cognitive and etiologic criteria for MCI due to AD, and is also ApoE-4 positive, is more likely to progress to AD within a few years than an individual without this genetic characteristic [8].

SYNAPTIC FAILURE:

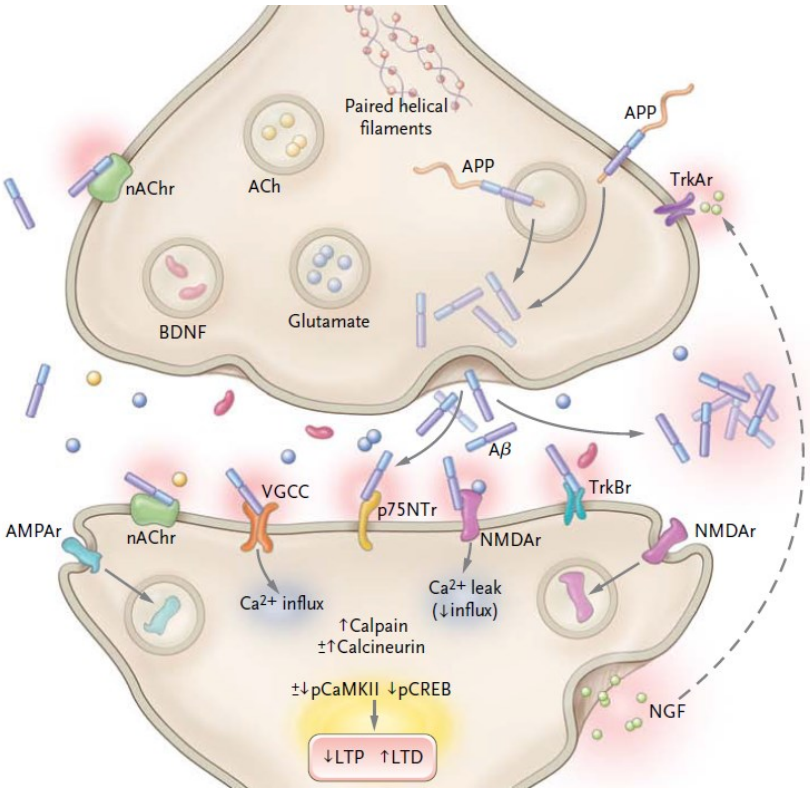

Figure 4: Synaptic Dysfunction in Alzheimer's disease

(Source: Querfurth Henry W, LaFerla Frank M. Review Article: Alzheimer's disease. N Engl J Med 2010; 362:329-344 DOI: 10.1056/NEJMra0909142)

Alzheimer's disease may be primarily a disorder of synaptic failure. Synaptic loss is the best pathological correlate of cognitive impairment in subjects with AD. The relation between synaptic activity and Aβ in AD pathogenesis is an area that is widely discussed.

Chronic synaptic activity could be detrimental since addition of extracellular amyloid beta alters synapses and impairs synaptic plasticity. Hippocampal synapses begin to decline in patients with mild cognitive impairment in whom remaining synaptic profiles show compensatory increases in size [9]. In mild Alzheimer's disease, there is a reduction of about 25% in the presynaptic vesicle protein synaptophysin. With advancing disease, synapses are disproportionately lost relative to neurons, and this loss is the best correlate with dementia. Aging itself causes synaptic loss which particularly affects the dentate region of the hippocampus [1].

KEY PROTEINS:

1. TOMM40

Translocase of outer mitochondrial membrane 40 homolog (yeast), also known as TOMM40, is a protein which in humans is encoded by the *TOMM40* gene. The protein encoded by this gene is localized in the outer membrane of the mitochondria. It is the channel-forming subunit of the translocase of the mitochondrial outer membrane (TOM) complex that is essential for import of protein precursors into mitochondria. *TOMM40* codes for a protein that is embedded into outer membranes of mitochondria and is required for the movement of proteins into mitochondria. More precisely, TOMM40 is the channel-forming subunit of a translocase of the mitochondrial outer membrane (TOM) that is essential for protein transport into mitochondria [10, 11, 12]

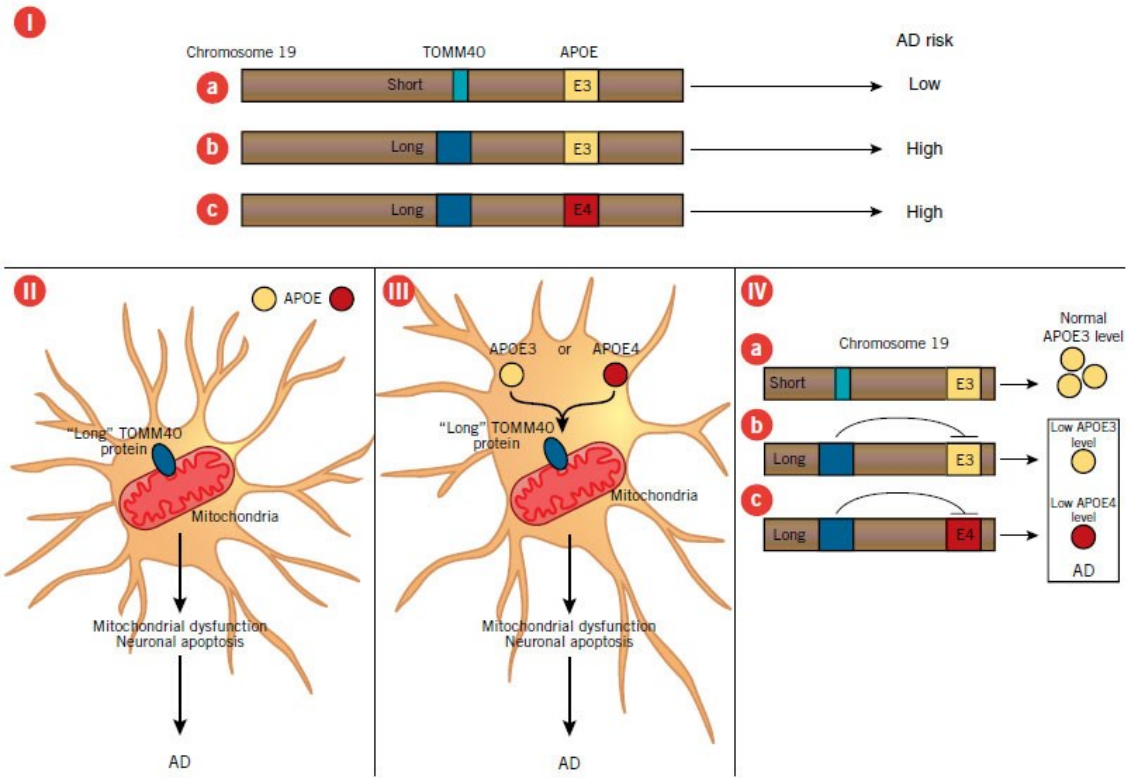

Figure 5: Role of TOMM40 in Alzheimer's disease

(Source: Osherovich, L. *SciBX* **2**(24); doi:10.1038/scibx.2009.962)

The TOMM40 gene, which encodes a mitochondrial protein, lies on chromosome 19 adjacent to the gene encoding apolipoprotein E (APOE), a well-known AD risk factor. DNA sequencing of 340 individuals revealed two forms of a noncoding region within the TOMM40 gene.

(**I**) The most common form contains a short sequence repeat and correlates with an average age of AD onset of 78 years in carriers of the AD-neutral APOE allele E3 [**a**], whereas an alternative, longer repeat sequence correlates with average age of AD onset of 70 years in E3 carriers (n=105, $p<0.03$) [**b**]. The long form is also present in nearly all

carriers of APOEallele E4 [**c**], which was previously shown by Roses to correlate with early AD onset.

(**II**) APOE functions outside of the neuron, physically separated from an abnormal form of TOMM40. Abnormal TOMM40 could lead to mitochondrial dysfunction, apoptosis and eventually AD in an APOE-independent manner.

(**III**) Alternatively, intracellular APOE protein and TOMM40 protein could interact at the mitochondrial surface to disrupt mitochondrial metabolism, leading to apoptosis and AD.

(**IV**) The length variation in the TOMM40 gene could influence the expression of the adjacent APOE gene. The short form of TOMM40 could lead to strong transcription of APOE and thus normal E3 protein levels that protect against disease [**a**]. However, the long form of TOMM40 could dampen expression of E3 [**b**] or E4 [**c**], leading to inadequate APOE protein levels and subsequently to AD.

In humans, certain alleles of this gene have been statistically associated with an increased risk of developing late-onset Alzheimer's disease. One study has found that TOMM40 risk alleles appears twice as often in people with Alzheimer's disease than those without it. Because *TOMM40* is located on chromosome 19, and is closely adjacent to *APOE*, another gene known to be associated with Alzheimer's, another study has suggested that the statistically significant correlation of TOMM40 with Alzheimer's is due to linkage disequilibrium [13, 14,15,16]

2. SYNAPTOPHYSIN

Synaptophysin, also known as the major synaptic vesicle protein p38, is a protein that in humans is encoded by the *SYP* gene. The encoded protein has 313 amino acids with a predicted molecular weight of 38 kDa [17]. It is present in neuroendocrine cells and in virtually all neurons in the brain and spinal cord that participate in synaptic transmission. It acts as a marker for neuroendocrine tumors, and its ubiquity at the synapse has led to the use of synaptophysin immunostaining for quantification of synapses [18].

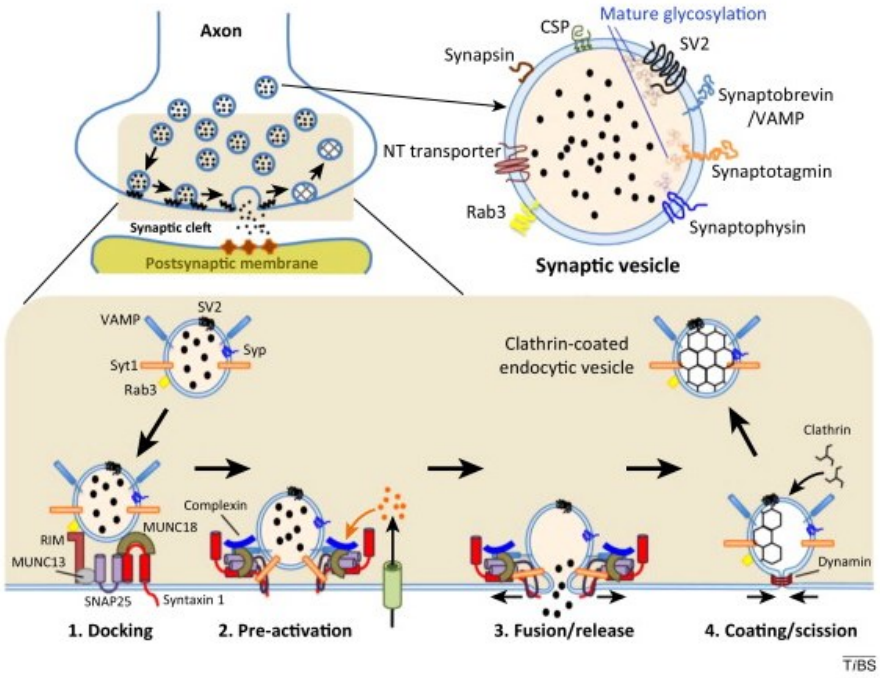

Figure 6: Possible role of synpatophysin in neurodegenerative disorders

(Source:Trends Biochem Sci. 2015 July; 40(7): 385–396. Published online 2015 April 30. doi: 10.1016/j.tibs.2015.03.015)

Synaptophysin was the first synaptic vesicle protein to be cloned and characterized and is known to belong to a family of proteins with 4 transmembrane domains that includes synaptogyrin and synaptoporin. Syp is the most abundant SV protein by mass, accounting for ~10 % of total vesicle protein. Because syp is exclusively localized to SVs, it is widely used as a marker for pre-synaptic terminals [19].

3. COX-IV

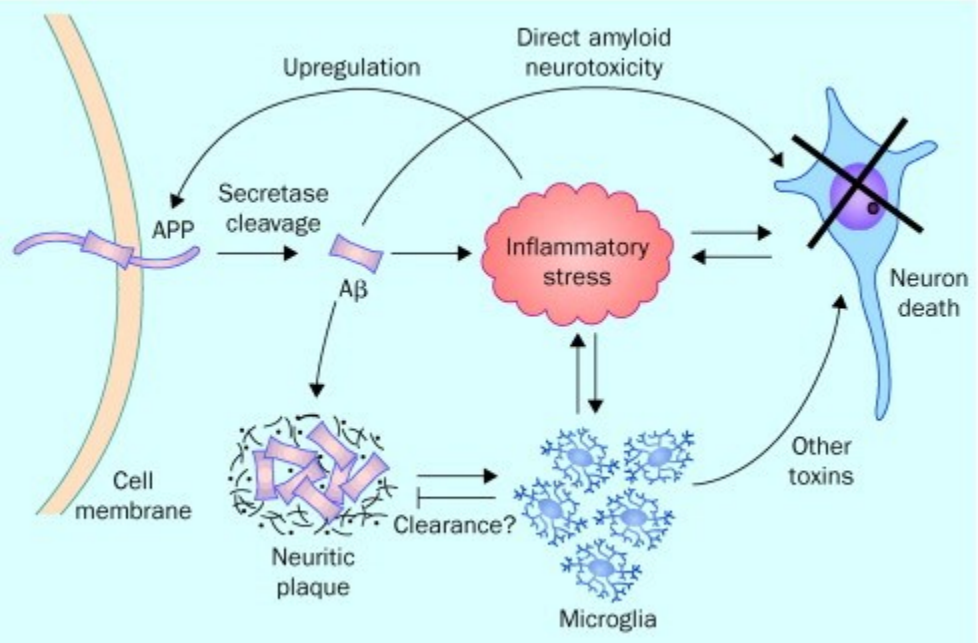

Figure 7: Inflammatory stress in Alzheimer's disease

(Source: Aisen, Paul S. The potential of anti-inflammatory drugs for the treatment of Alzheimer's disease. The Lancet Neurology , Volume 1 , Issue 5 , 279 – 284)

Cytochrome c oxidase (COX) is a heterooligomeric enzyme consisting of 13 subunits localized to the inner mitochondrial membrane. It is the terminal enzyme complex in the respiratory chain, catalyzing the reduction of molecular oxygen to water coupled to the translocation of protons across the mitochondrial inner membrane to drive ATP synthesis.

The 3 largest subunits forming the catalytic core are encoded by mitochondrial DNA while the other smaller subunits, including COX IV, are nuclear-encoded [20]. Deficiency in COX activity has been correlated with a number of human diseases. This protein is one of the nuclear-coded polypeptide chains of cytochrome c oxidase, the terminal oxidase in mitochondrial electron transport [21].

4. PSD95

PSD-95 (Post Synaptic Density Protein 95) also known as SAP-90 (synapse-associated protein 90) is a protein that in humans is encoded by the *DLG4* (discs large homolog 4) gene. PSD-95 is a member of the membrane-associated guanylate kinase (MAGUK) family. PSD-95 a membrane associated guanylate kinase (MAGUK) scaffolding protein located in neural postsynaptic densities. Like all MAGUK-family proteins, its basic structure includes three PDZ domains, an SH3 domain, and a guanylate kinase-like domain (GK) connected by disordered linker regions. It is almost exclusively located in the post synaptic density of neurons and is involved in anchoring synaptic proteins. Its direct and indirect binding partners include neuroligin, NMDA receptors, AMPA receptors, and potassium channels. It plays an important role in synaptic plasticity and the stabilization of synaptic changes during long-term potentiation.

It is required for synaptic plasticity associated with NMDA receptor signaling. Its overexpression or depletion changes the ratio of excitatory to inhibitory synapses in hippocampal neurons. High levels in postsynaptic density of neurons in the forebrain. Also in presynaptic region of inhibitory synapses formed by cerebellar basket cells on axon hillocks of Purkinje cells [22, 23].

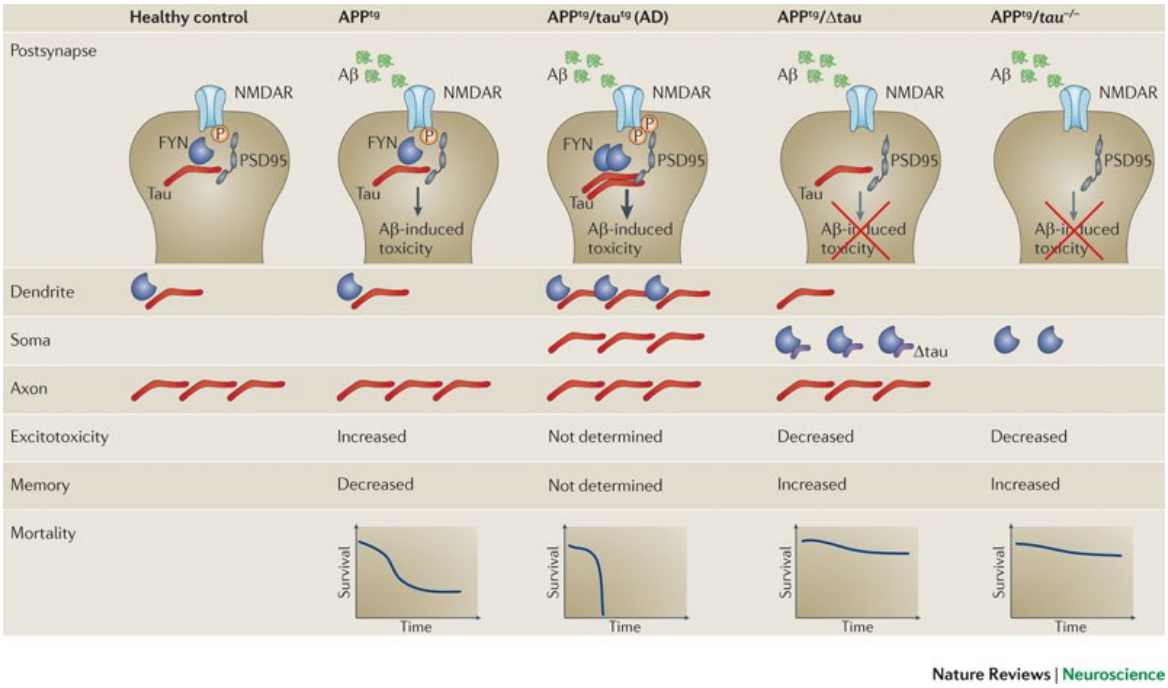

Figure 8: Role of PSD95 in Alzheimer's disease

(Source: Lars M. Ittner & Jürgen Götz. Amyloid-β and tau — a toxic pas de deux in Alzheimer's disease. Nature Reviews Neuroscience 12, 67-72 (February 2011) doi:10.1038/nrn2967)

N-methyl-D-aspartate (NMDA) receptor-evoked excitotoxicity contributes to region-specific loss of glutamatergic synapses responsible for cognitive decline in AD [24].

MATERIALS AND METHODS

In order to develop and optimize an extraction protocol for quantitative proteomic studies to investigate key proteins involved in oxidative pathways in Alzheimer's disease, we used mouse brain tissue followed by human brain tissue samples. Human tissue samples were obtained from the Baltimore Longitudinal Study of Aging (BLSA) and were categorized by the Consortium to Establish a Registry for Alzheimer's disease (CERAD) - established in 1986 by a grant from the National Institute on Aging (NIA), to standardize procedures for the evaluation and diagnosis of patients with Alzheimer's disease (AD). Patients and non-demented control subjects were recruited from 24 NIA-sponsored Alzheimer's disease Research Centers and other university programs in the US.

1. CHEMICALS AND REAGENTS

a. RIPA Buffer

RIPA buffer derives its name from the original application for which it was developed: the radio-immunoprecipitation assay. RIPA cell lysis reagent is highly effective for protein extraction from a variety of cell types because it contains three non-ionic and ionic detergents. It is a sensitive assay using radiolabeled antigens to detect specific antibodies in serum. RIPA Buffer does not contain protease or phosphatase inhibitors. It enables efficient cell lysis and protein solubilization while avoiding protein degradation and interference with the proteins′ immunoreactivity and biological activity. It also results in low background in immunoprecipitation and molecular pull-down assays. It is suitable for small scale affinity pull-down assays such as

immunoprecipitation and for western blotting. It minimizes non-specific protein binding interactions to ensure low background. This buffer is more denaturing than NP-40 or Triton X-100 lysis buffer because it contains the ionic detergents SDS and sodium deoxycholate as active constituents and is particularly useful for disruption of nuclear membranes in the preparation of nuclear extracts. The antigens are allowed to react with the serum and then precipitated using a special reagent such as protein A sepharose beads. The bound radiolabeled immunoprecipitate is then commonly analyzed by gel electrophoresis [25, 26].

RIPA buffer's rapid and efficient cell lysis and solubilization of a wide range of proteins, including cytoplasmic, membrane and nuclear proteins, makes it a standard for Western blotting. When protein quantitation is desired, RIPA buffer is the lysis buffer of choice due to its compatibility with the BCA Protein Assay, although it can denature kinases and can disrupt protein-protein interactions in immunoprecipitation/pull down assays

Composition: 50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS.

b. Urea Buffer

Urea buffer is another versatile and efficient cell and tissue lysing buffer whose typical composition includes: TRIS base 40 mM, Urea 7 M, Thiourea 2 M, NP-40 or CHAPS 4%, DTT 10 mM. Urea is used at concentrations ranging from 5 to 9 M, often with thiourea at concentrations up to 2 M. The additive thiourea can dramatically enhance the solubility of a wide range of proteins – nuclear, membrane, cytosolic, and including even tubulin that is highly prone to aggregation, in urea buffer. Urea inactivates proteases that

degrade cellular proteins. Therefore, there is little need to add protease inhibitors. However, urea and thiourea can hydrolyze to cyanate and thiocyanate, respectively, which can modify amino groups on proteins, (e.g. carbamylation of proteins by isocyanate), and this hydrolysis is promoted by heat [27].

2. BRAIN SAMPLES

a. Mouse brain tissue – 25 to 50 mg of mouse brain tissue was used with RIPA or urea buffer followed by sonication and/or homogenization.
b. Human brain tissue –We categorized our samples from the superior and middle temporal gyrus (SMTG) and middle frontal gyrus (MFG) into three groups:

Three postmortem groups were studied: controls with normal and stable cognition; cognitively intact subjects with senile plaque densities diagnostic for possible AD (p-AD) and neurofibrillary changes characteristic of early AD; and individuals with definite AD and neurofibrillary changes typical of incipient to severe AD

Normal: At any age, persons may potentially be free of objective or subjective symptoms of cognition and functional decline and also free of associated behavioral and mood changes.

MCI: An intermediate stage between the expected cognitive decline of normal aging and the more pronounced decline of dementia. Memory deficits in MCI may remain stable for years.

AD: Using standardized diagnostic criteria and assessment instruments, CERAD subjects were examined at entry and annually thereafter, to observe the natural progression of AD.

Autopsy examination of the brain was included, to obtain neuropathologic confirmation of the clinical diagnosis. Brain tissue was homogenized in liquid nitrogen and the brain powder was stored at −80 °C.

3. PROTEIN EXTRACTION

Homogenization:

In cell biology or molecular biology research, homogenization is a process whereby a biological sample is brought to a state such that all fractions of the sample are equal in composition, i.e. a homogenized sample is mixed so well that removing some of the sample does not alter the overall molecular make-up of the sample remaining, and is identical to the fraction removed. Homogenization in biology is often followed by or combined with, cell lysis and/or molecular extraction [28].

Purification of protein from animal and human tissues using TissueLyser LT:

Placed 2 ml microcentrifuge tubes containing 1 stainless steel bead (5 mm mean diameter) on dry ice for at least 15 minutes. Kept the insert of the TissueLyser LT Adapter at room temperature. Transferred up to 30 mg fresh or frozen tissue to the pre-cooled tubes and incubated for another 15 minutes on dry ice. Placed the tubes into the insert of the TissueLyser LT Adapter and incubated at room temperature for 2 minutes to avoid freezing of lysis buffer. Incubating for longer than 2 minutes results in potential protein degradation. Immediately added the appropriate volume of lysis buffer to each tube. Placed the insert with sample tubes into the base of the TissueLyser LT Adaptor,

which is attached to the TissueLyser LT. Operated the TissueLyser LT for 2-5 minutes at 50 Hz until no tissue debris was visible.

Sonication:

Sonication is a process in which sound waves are used to agitate particles in solution. The sound waves used in sonication are usually ultrasound waves with frequencies above above 20 kHz and as frequency increases the strength of the agitation increases. In solution, the particles vibrate because as they experience cycles of pressure, microscopic vacuum bubbles form and then collapse into solution, a process called cavitation. These vibrations can disrupt molecular interactions, break clumps of particles apart, and lead to mixing. In the case of dissolved gas, these vibrations can allow the gas bubbles to come together and more easily leave the solution. Indirect sonication is most effective for very small samples because foaming and sample loss are eliminated.

Protein extraction from tissues using Bioruptor:

Used the Bioruptor Standard to sonicate the brain tissue, adding ice-cold lysis buffer. Proceeded to sonicate with the following settings:

Power: H position (High)

Sonication cycle: 30 sec ON/30 sec OFF

Total sonication time: 5-10 cycles

Temperature: 4°C

Vortexed samples after every 5 cycles and visually inspected them to find that they were in soluton and viscosity had reduced. Transferred the supernatant to a new tube and

centrifuged samples at 14,000 rpm for 15 min at 4°C to remove any remaining insoluble material. Stored protein extracts at -80°C.

4. PROTEIN QUANTIFICATION

Lowry Assay:

Under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue.

The Bio-Rad DC Protein Assay is a colorimetric assay for protein concentration. The reaction reaches 90% of its maximum color development within 15 minutes thereby saving valuable time and the color changes not more than 5% in 1 hour or 10% in 2 hours after the addition of reagents. Proteins effect a reduction of the Folin reagent by loss of 1, 2, or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue color with maximum absorbance at 750 nm and minimum absorbance at 405 nm.

1. Preparation of Working Reagent: Added 20 µl of reagent S to each ml of reagent A needed for the run.
2. Preparation of a standard curve: A standard curve was prepared each time the assay was performed.

a. Prepared 6 dilutions of the protein standard from 0.1 mg/mL to 1 mg/mL protein.
b. Sample Calculation: 1430 µg in 1000 µL

$$100\ \mu g = \frac{100*1000}{1430} = 69.93\ \mu L \text{ of Standard} \qquad (100\mu g = 0.1mg)$$

c. Made it up to 1000 µL with the buffer (Triton X-100 buffer)

Sr. No.	Conc(mg/mL)	Vol.of stock (µL)	Vol. of Triton-X-100 buffer (µL)
1	0.1	69.93	930.07
2	0.2	139.86	860.14
3	0.4	279.72	720.28
4	0.6	419.58	580.42
5	0.8	559.44	440.56
6	1	699.30	300.70

Table 1: Sample calculation table to perform the Lowry Assay

Sample Calculation: 69.93 µL of standard + 930.07 µL of Buffer (For a concentration of 100µg)

d. Pipetted 100µL of the above into clean, dry test tubes.

e. Added 500 µL of working reagent (A+S prepared before) into each test tube. Vortex.

f. Added 4.0 mL of reagent B into each test tube and vortexed immediately.

g. After 15 minutes, absorbances were read at 750 nm using the Smart Spec Plus.

Bradford Assay:

The Bradford protein assay is a colorimetric protein assay originally described by Marion Bradford which uses a disulfonated tripheynlmethane compound called Coomassie Brilliant Blue G-250 (CBB G-250). Under acidic conditions the protein binds to mostly basic amino acids such as arginine, lysine and other amino acids including histidine. Under these conditions, dye-amino acid binding results in a shift dye pKa of the dye causing the color to change from green/brown tint to blue. The protein-bound dye is measured at 595 nm. The more protein, the more intense the blue color. Amino acid composition as well as post-translational modification of proteins can lead to variation of dye intensity. The amount of absorbance of the bound dye at 595 nm is linearly related to the concentration of the protein.

5. ANTIBODIES

Antibody	**Host**	**Clonality**	**Immunogen**	**Molecular Weight**	**Isotype**
TOMM40	Rabbit	Polyclonal	Recombinant protein of human TOMM40	38 kDa	IgG
Synaptophysin	Mouse	Monoclonal	Electrophoretically purified synaptophysin	38 kDa	IgG1
COX-IV	Rabbit	Polyclonal		15 kDa	IgG
PSD95	Rabbit	Polyclonal	Synthetic peptide conjugated to KLH derived from within residues 50-150 of Mouse PSD95	95 kDa	IgG

Table 2: Characteristics of antibodies used in experiments

6. PEPTIDES

Peptide	Nature	Source	Species	Amino acids	Concentration	Molecular Weight
Synaptophysin	Recombinant	Wheat Germ	Human	1-313	2 µg at 0.05 mg/ml	61 kDa including tags
COX-IV	Recombinant	Wheat Germ	Human	1-169	2 µg at 0.05 mg/ml	44 kDa including tags

Table 3: Characteristics of peptides used in experiments

7. WESTERN BLOTS

The term "blotting" refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. Western blotting, also called immunoblotting because an antibody is used to specifically detect its antigen, was introduced by Towbin, et al. in 1979 and is now a routine technique for protein analysis. The specificity of the antibody-antigen interaction enables a target protein to be identified in the midst of a complex protein mixture. Western blotting can produce qualitative and semi-quantitative data about that protein.

The most sensitive detection methods use a chemiluminescent substrate that, when combined with the enzyme, produces light as a byproduct. The light output can be captured using film, a CCD camera or a phosphorimager that is designed for chemiluminescent detection. Alternatively, fluorescently tagged antibodies can be used, which are directly detected with the aid of a fluorescence imaging system. Whatever

system is used, the intensity of the signal should correlate with the abundance of the antigen on the membrane.

DIRECT METHOD:

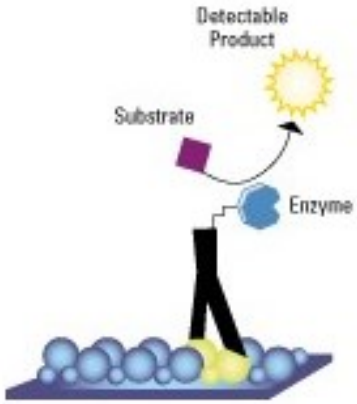

Figure 9: Principle of using the direct method in Western Blots

(Source: ThermoFisher Scientific: Western Blotting Handbook)

This method is quick since only one antibody is used. There is no concern for cross-reactivity of a secondary antibody. Labeling may reduce immunoreactivity of primary antibody. The labeled primary antibodies are expensive and there is low flexibility in choice of primary antibody label with little signal amplification.

INDIRECT METHOD:

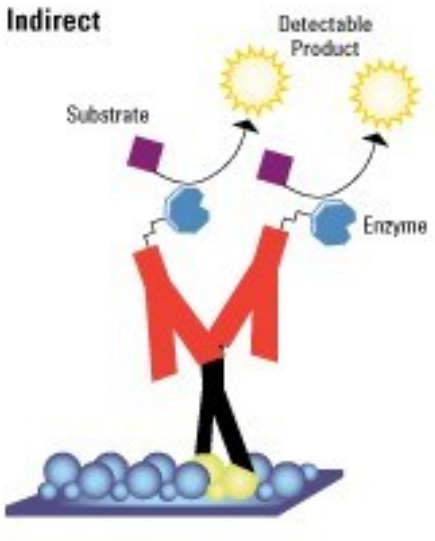

Figure 10: Principle of using the indirect method in Western Blots

(Source: ThermoFisher Scientific: Western Blotting Handbook)

Here the secondary antibody can amplify signal and a variety of labeled secondary antibodies are available. One secondary may be used with many primary antibodies Labeling does not affect primary antibody immunoreactivity and changing secondary allows change of detection method. However, secondary antibodies may produce nonspecific staining

Western Optimization:

- Prepared samples in lysis buffer to concentration of lowest sample. Added 20 uL of mixed 2x Laemmli and beta mercaptonal sample buffer (prepare by adding 50 uL beta mercaptonal to 950 uL laemmli buffer) to each sample. Votex samples. The amount of sample to prepare depends on the size of the well for the gel.

- Denatured samples by putting in Fisher Dry Bath at 100ºC for 5 minutes.

- Placed gel in electrophoresis container with 1X running buffer. Ran at 225V. (4–20% Criterion™ TGX™ Gel, 12+2 well, 45 µl)

- Prepared nitrocellulose membrane and filter paper stacks. Moistened Nitrocellulose and filter paper in 1X transfer buffer for 30 minutes.

- Set up Trans Blot – Midi gel (2.5A, 25V, 7 min)

- Placed in tray of water for 10 minutes

- Placed membrane in appropriately sized cassette and added blocking buffer until the membrane is covered. Closed cassette and put on shaker for 1 hour.

- Prepared 10 mL primary antibody solution in blocking buffer to desired concentration. Placed on shaker for 1 hour using surface transfer mechanisms.

- Washed with blocking buffer (or wash buffer) 3 times for 5 min each on shaker at room temperature.

- Prepared 10 mL secondary antibody solution in blocking buffer. These antibodies are sensitive to light so wrapped the cassette in in foil while incubating for 1 hour.

- Washed with blocking buffer 6 times for 5 min each on shaker at room temperature.

- Mixed ECL reagents - 6 mL of one and 6 mL of the other. Did not mix until right before use. Added to the membrane – 5 minutes.

- Imaged membrane dry

RESULTS AND DISCUSSION

1. STANDARD CURVES

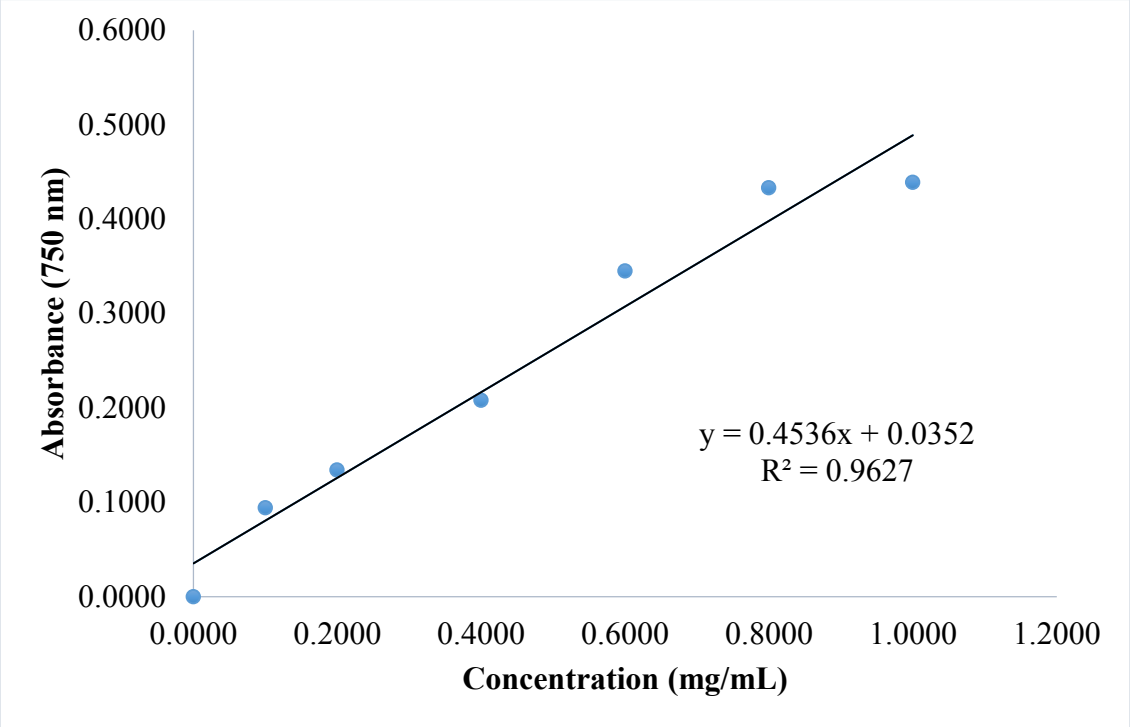

Figure 11: Typical absorbance plot for BSA using a spectrophotometer procedure – Lowry Assay at 750 nm.

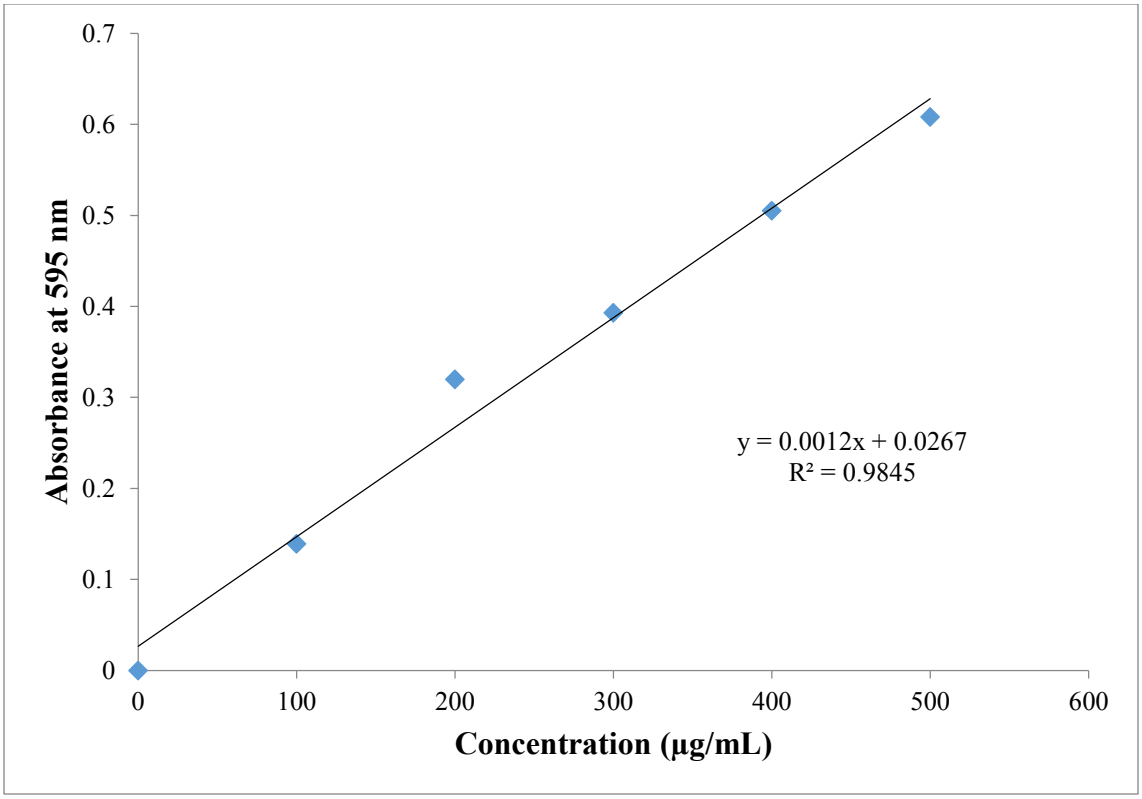

Figure 12: Typical absorbance plot for BSA using a microplate procedure – Bradford Assay at 595 nm

Determinations are made with a high level of confidence (r2 = 0.98). Determinations in the lower portion of the curve offer the greatest degree of accuracy with a polynomial fit due to the greater change in signal verses change in protein concentration.

2. LYSIS

Different approaches to extraction using homogenization (H) and/or sonication(S) with RIPA and/or Urea buffers were attempted on mice brain samples and total protein concentration was calculated using the Bradford Assay.

Sr. No	Sample	Buffer	Conc. (µg/mL)
1.	A	RIPA	47
2.	B	Urea	699
3.	C	RIPA	149
4.	D	Urea	647
5.	E	RIPA	46
6.	F	Urea	636

Table 4: Optimization Steps: Mortar & Pestle – Sonication – Homogenization – Centrifugation

Sr. No	Sample	Buffer	Conc. (µg/mL)
1.	G	RIPA	26
2.	H	Urea	689
3.	I	RIPA	42
4.	J	Urea	615
5.	K	RIPA	41
6.	L	Urea	621

Table 5: Optimization Steps: Mortar & Pestle – Homogenization – Centrifugation

Sample	Buffer	Method	Amount (mg)	Total Protein (µg/mL)
1	RIPA	S	50	1301.857
2	RIPA	H	50	426.159
3	**RIPA**	**S+H**	**50**	**1321.339**
4	RIPA	S	25	1294.484
5	RIPA	H	25	332.493
6	RIPA	S+H	25	1273.665
7	Urea	S	50	992.284
8	Urea	H	50	992.207
9	Urea	S+H	50	1085.339
10	Urea	S	25	1021.736
11	Urea	H	25	958.286
12	Urea	S+H	25	955.230

Table 6: Final Optimization of extraction protocol

3. PROTEIN CONCENTRATIONS FROM DIFFERENT EXTRACTION FRACTIONS
C = cellular, E = extracellular, I = insoluble, R = other.

Sr. No.	SMTG C (µG/mL)	SMTG E (µG/mL)	SMTG I (µG/mL)	SMTG R (µG/mL)
1	11	81	24	73
2	18	99	31	95
3	21	37	19	28
4	60	70	13	61
5	42	69	33	55
6	70	98	53	60
7	29	71	40	63
8	41	73	67	59

Table 7: **Protein concentrations from different extraction regions of Normal CERAD-B category patients.** Highest concentration was found in samples from Extracellular regions and lowest varied between Cellular and Insoluble fractions.

Sr. No.	SMTG C (µG/mL)	SMTG E (µG/mL)	SMTG I (µG/mL)	SMTG R (µG/mL)
1	43	145	53	38
2	202	216	117	198
3	185	189	242	188
4	178	249	173	174
5	185	249	119	150
6	187	218	109	160
7	110	220	56	108

Table 8: Protein concentrations from different extraction regions of MCI category patients. Highest concentrations were found in Extracellular fractions. Lowest concentrations were seen predominantly in the Insoluble fractions.

Sr. No.	SMTG C (µG/mL)	SMTG E (µG/mL)	SMTG I (µG/mL)	SMTG R (µG/mL)
1	149	161	50	143
2	65	173	32	50
3	53	98	51	73
4	91	101	74	85
5	117	147	114	136
6	99	153	87	147
7	106	160	39	56
8	111	181	68	128
9	97	114	75	101

Table 9: Total protein concentrations from different extraction regions of Demented CERAD-B category patients. Highest concentrations were found in Extracellular fractions. Lowest concentrations were seen in the intracellular fractions.

Sr. No.	SMTG C (µG/mL)	SMTG E (µG/mL)	SMTG I (µG/mL)	SMTG R (µG/mL)
1	31	51	7	42
2	26	47	6	41
3	58	79	48	61
4	73	87	34	59
5	76	91	56	82
6	88	94	46	71
7	63	78	32	72
8	42	53	37	49
9	66	81	43	58

Table 10: Protein concentrations from different extraction regions of Demented CERAD-C category patients. Highest concentrations were found in Extracellular fractions. Lowest concentrations were seen in the intracellular fractions.

Patient Category	**Cellular (µg/mL)**	**Extracellular (µg/mL)**	**Insoluble fraction (µg/mL)**
NORMAL	18	99	31
NORMAL	70	98	53
MCI	178	249	173
MCI	110	220	56
AD	76	91	56
AD	88	94	46

Table 11: Summary of Protein Concentrations: MCI patients have larger total protein content than the other patient groups.

4. ANTIBODY CONCENTRATIONS

Sr. No	Name of the Antibody	Actual Primary Antibody concentration	Primary Antibody concentration used	Primary Antibody volume (µL)	Secondary Antibody concentration	Secondary Antibody volume (µL)	Volume of Blocking Buffer (mL)
1	TOMM40	1:5000	1:1750	20 µL	1:5000	7 µL	35
2	Synaptophysin	1:10000	1:3333	3 µL	1:1000	10 µL	10
4	COX IV	1:1000	1:1000	10 µL	1:5000	2 µL	10

Table 12: Concentrations and volumes of antibodies used in experiments

5. WESTERN BLOTS

a. Using mice brain tissue

(i) TOMM40

Figure 13: Western Blot Analysis of mice brain samples using RIPA (R) and urea (U) buffer with TOMM40 antibody of primary and secondary concentration of 1:5000. TOMM40 has a molecular weight of 38 kDa

(ii) SYNAPTOPHYSIN

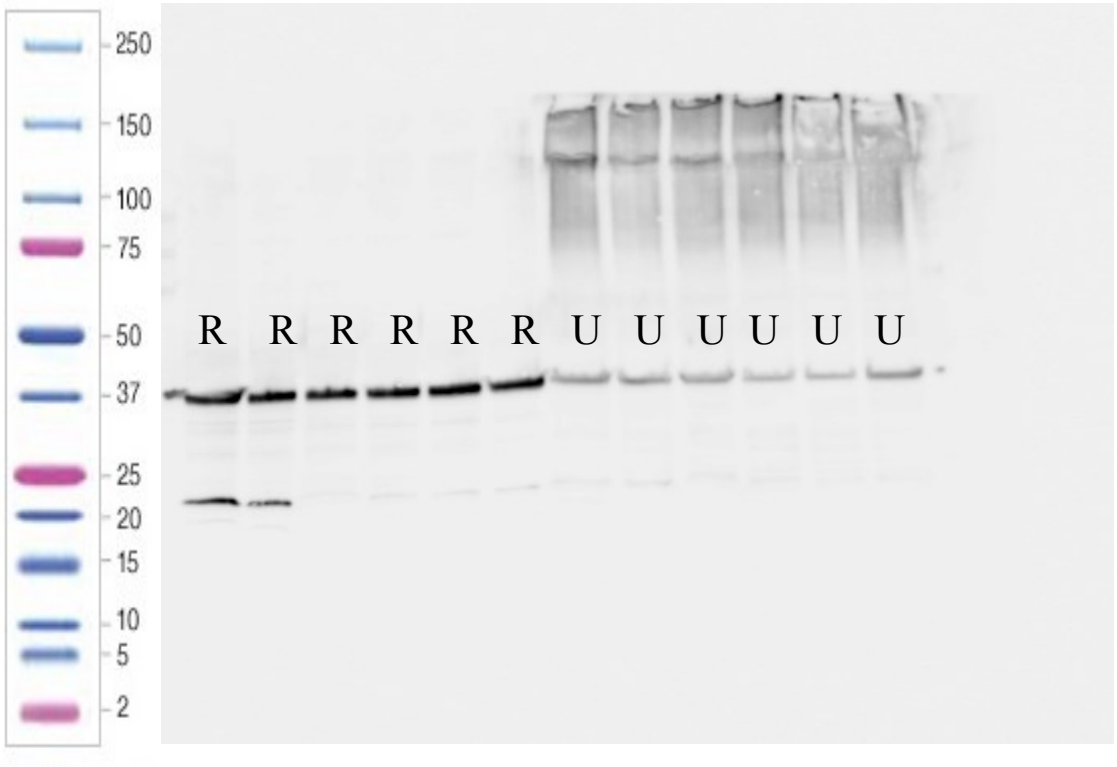

Figure 14: Western Blot Analysis of mice brain samples using RIPA (R) and urea (U) buffer with synaptophysin antibody of primary concentration= 1:10000 and secondary concentration of 1:1000. Synaptophysin has a molecular weight of 38 kDa.

(iii) COX-IV

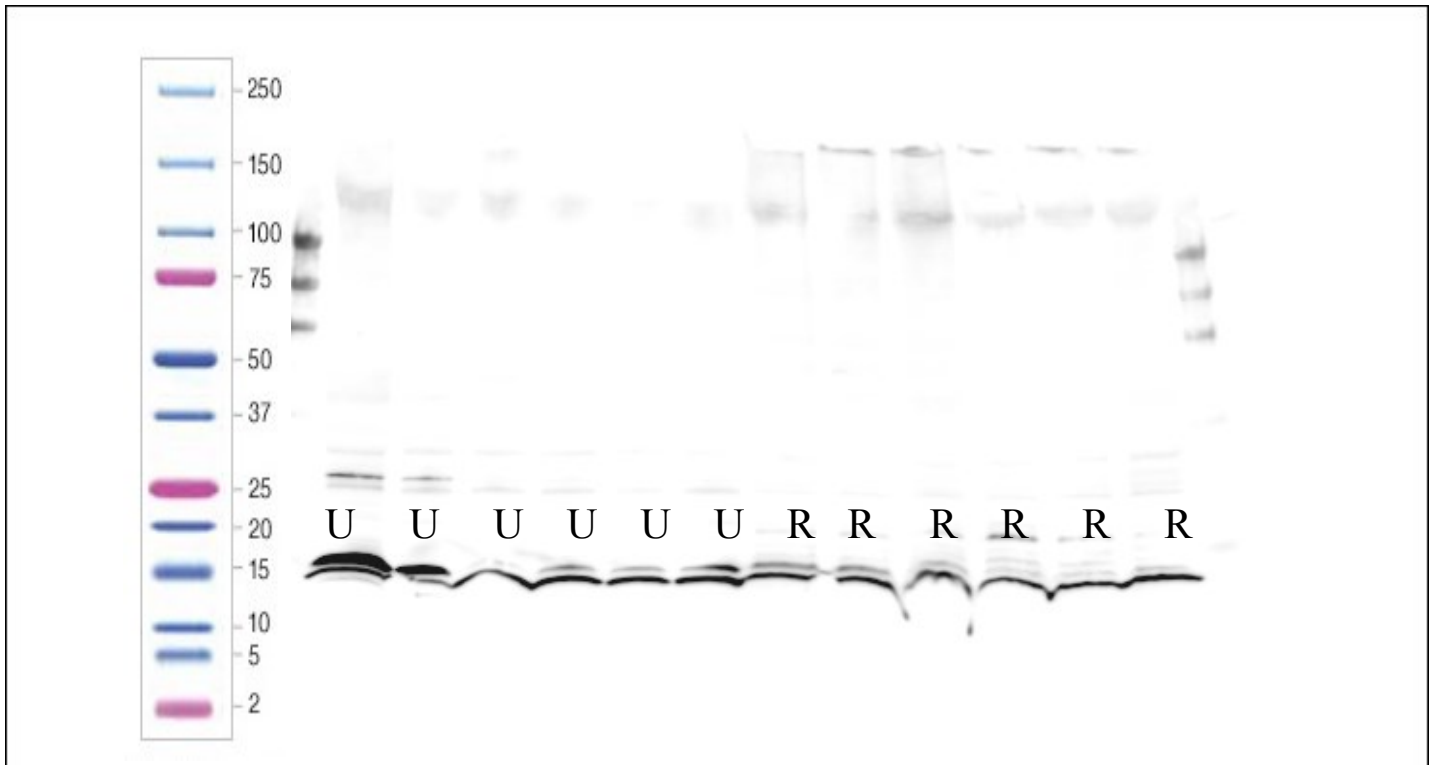

Figure 15: Western Blot Analysis of mice brain samples using RIPA (R) and urea (U) buffer with COX-IV antibody of primary concentration= 1:1000 and secondary concentration of 1:2000. COX-IV has a molecular weight of 15 kDa.

b. Using human brain tissue

(i) TOMM40

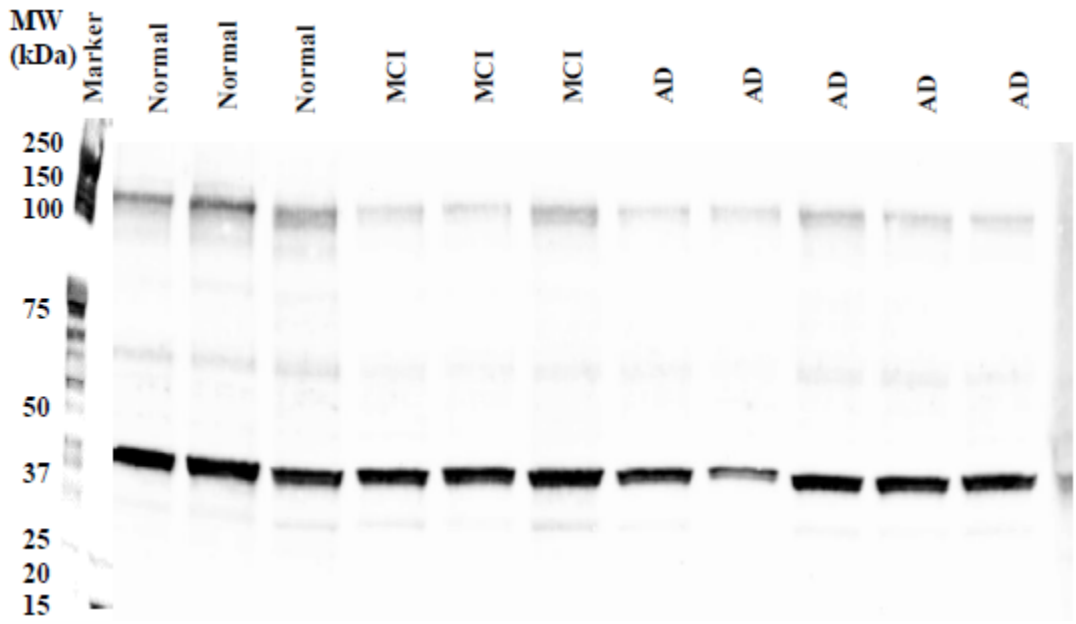

Figure 16: Western Blot Analysis of mice brain samples from patient categories: Normal (N), MCI (M), AD from CERAD-B and CERAD-C using RIPA buffer with TOMM40 antibody of primary and secondary concentration of 1:5000. TOMM40 has a molecular weight of 38 kDa.

(ii) SYNAPTOPHYSIN

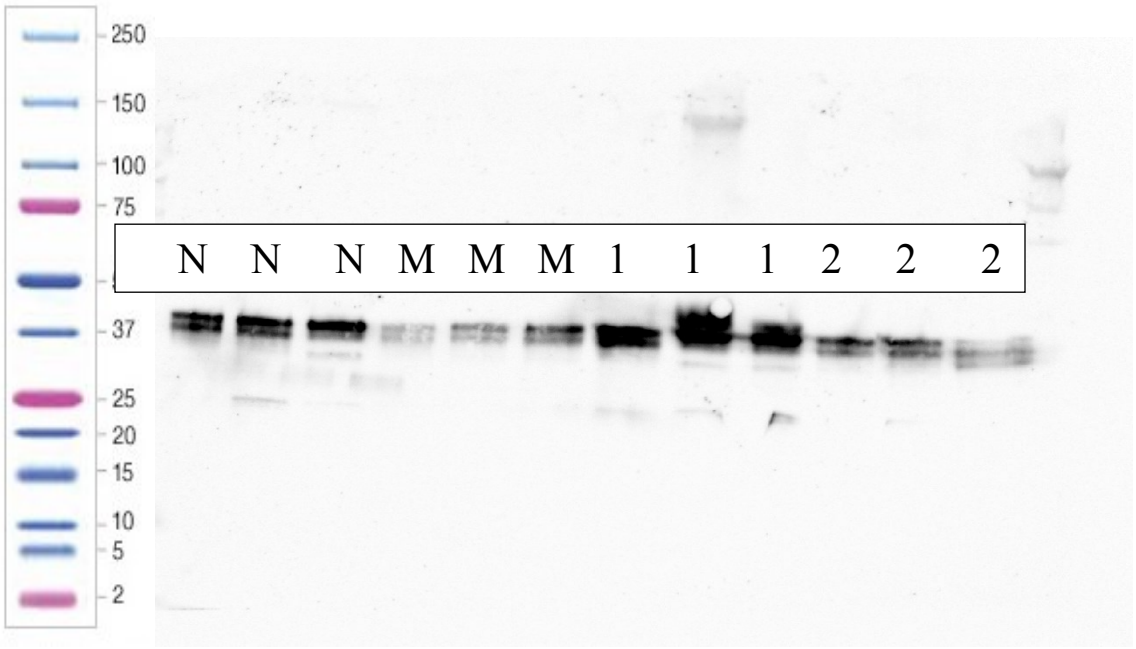

Figure 17: Western Blot Analysis of mice brain samples from patient categories: Normal (N), MCI (M), AD from CERAD-B (1) and CERAD-C (2) using RIPA buffer with synaptophysin antibody of primary concentration = 1:10000 and secondary concentration = 1:1000. Synaptophysin has a molecular weight of 38 kDa.

(iii) CYTOCHROME C OXIDASE- IV

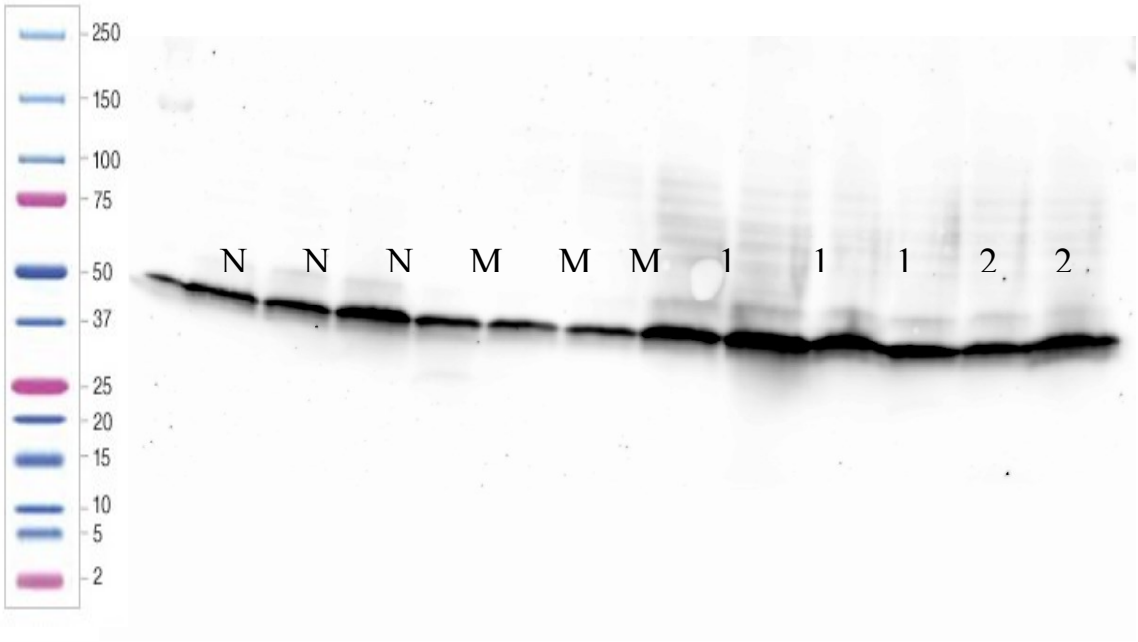

Figure 18: Western Blot Analysis of mice brain samples from patient categories: Normal (N), MCI (M), AD from CERAD-B (1) and CERAD-C (2) using RIPA buffer with COX-IV antibody of primary concentration = 1:1000 and secondary concentration = 1:2000. COX-IV has a molecular weight of 38 kDa.

c. Westerns with full length proteins

(i) SYNAPTOPHYSIN

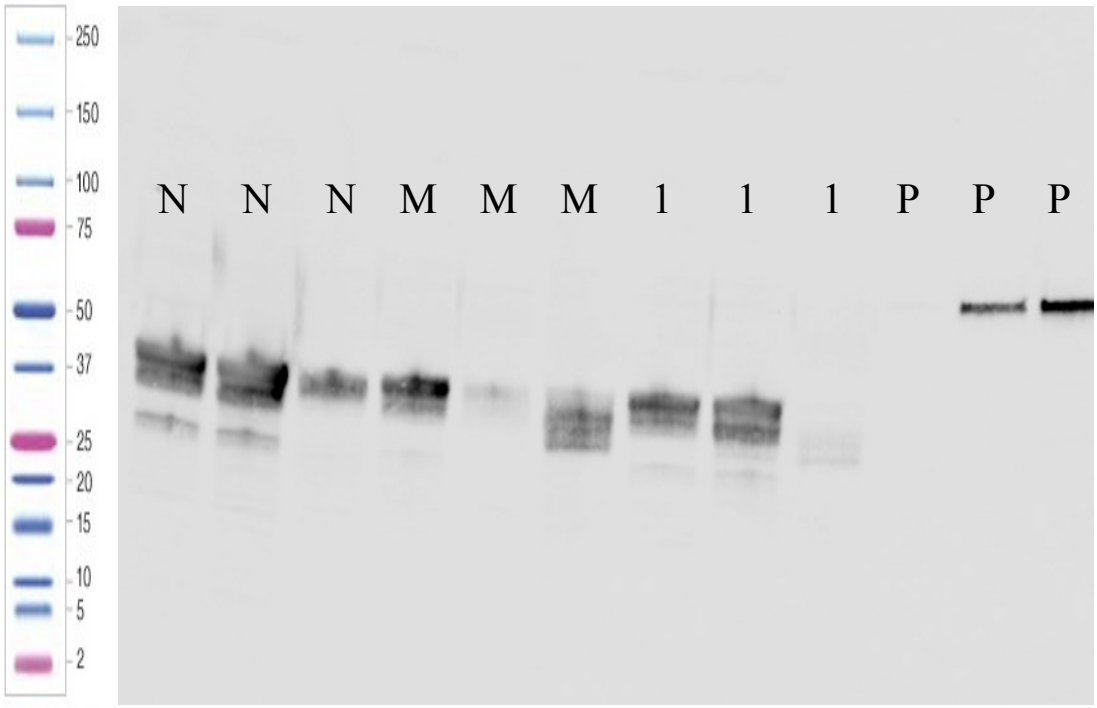

Figure 19: Western Blot Analysis of mice brain samples from patient categories: Normal (N), MCI (M), AD from CERAD-B (1) and full length synaptophysin peptide (P) using RIPA buffer with synaptophysin antibody of primary concentration = 1:10000 and secondary concentration = 1:1000. Synaptophysin has a molecular weight of 38 kDa.

(ii) COX –IV

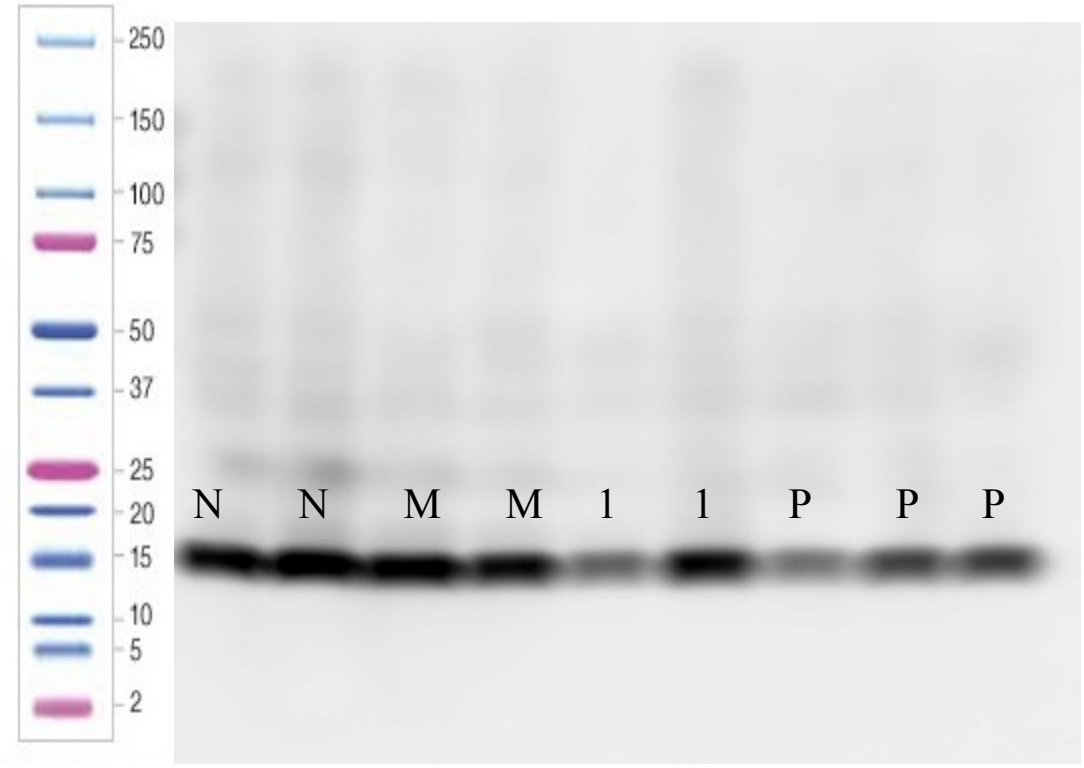

Figure 20: Western Blot Analysis of mice brain samples from patient categories: Normal (N), MCI (M), AD from CERAD-B (1) and using RIPA buffer with COX-IV antibody of primary concentration = 1:1000 and secondary concentration = 1:2000. COX-IV has a molecular weight of 38 kDa.

Comparison of protein concentration using Mass spectrometry across 3 patient groups will give us a better understanding of the roles of TOMM40, COX-1V, synaptophysin and PSD95 in AD, correlating temporal relationship of synaptic dysfunction to onset of cognitive dysfunction in AD patients.

d. Human brain tissue powdered using liquid nitrogen

(i) Protein Concentrations from Normal, MCI, Demented CERAD-B and CERAD-C patient categories.

Sample #	Category	Conc. (µg/mL)
1	NORMAL	142.26
2	NORMAL	60.268
3	NORMAL	32.599
4	NORMAL	184.82
5	NORMAL	750.81
6	NORMAL	1736.36
7	NORMAL	1906.25
8	MCI	1433.61
9	MCI	699.4
10	MCI	137.61
11	MCI	75.14
12	MCI	397.53
13	MCI	2066.45
14	MCI	1698.29
15	DEM. CERAD B	1463.55
16	DEM. CERAD B	1894.35
17	DEM. CERAD B	1461
18	DEM. CERAD B	347
19	DEM. CERAD B	41
20	DEM. CERAD C	377.78
21	DEM. CERAD C	1691.21
22	DEM. CERAD C	1969.55
23	DEM. CERAD C	1747.26
24	DEM. CERAD C	1733.04
25	DEM. CERAD C	1312.04
26	DEM. CERAD C	310.82

Table 13: Protein Concentrations from Normal, MCI, Demented CERAD-B and CERAD-C patient categories.

(ii) TOMM40

Figure 21: Western Blot Analysis of mice brain samples powdered using liquid nitrogen from patient categories: Normal (N), MCI (M), AD from CERAD-B (1) and CERAD-C (2) using RIPA buffer with TOMM40 antibody of primary and secondary concentration of 1:5000. TOMM40 has a molecular weight of 38 kDa.

While optimizing the protocol for best extraction of proteins from brain samples, it was clear that using a mortar and pestle prior to subjecting samples to homogenization yielded best results. Without initial grinding, the sonication and homogenization process did not take place smoothly and caused the equipment to only partially homogenize tissue. Using lab prepared Urea buffer with protease inhibitors and absorption at 750nm seemed to always yield similar total protein concentrations which was abnormal. This was rectified when no protease inhibitors were used. Urea buffer in Westerns showed high background. Proteins were precisely quantified against recombinant truncated protein standards. It was eventually seen that a combination of sonication and homogenization using the RIPA buffer yielded maximum total protein content using the Bradford assay at absorption 595nm. It was interesting to note that in all category of patients (Normal, MCI, CERAD-B and CERAD-C), highest protein concentrations were seen in extracellular fractions (E). In Normal patients, lowest concentrations varied between Cellular and Insoluble fractions. In MCI patients, least protein concentrations were seen predominantly in the insoluble fractions. The same result was observed in CERAD-B and CERAD-C patients as well. It was seen that MCI patients have the largest total protein concentrations among all patient groups. TOMM40, Synaptophysin and COX-IV were detected using preliminary Western Blots. PSD-95 was not detected in mice brain or human brain samples. Synaptophysin protein expression was markedly reduced in MCI patients which correlates correctly with the role of synaptophysin. Controls with full length proteins were used to make sure we are detecting the right protein in given patient categories and samples. Synaptophysin showed a marked increase in all normal patients which correctly correlates with its synaptic function. Cytochrome c oxidase (COX-IV)

showed increased concentrations in patients with Alzheimer's disease (CERAD-B and CERAD-C). TOMM40 showed a uniform concentration across all patient groups which negates its hypothesized function and role in Alzheimer's disease. Comparison of protein concentration using Mass spectrometry across three patient groups will give us a better understanding of the roles of TOMM40, COX-1V, synaptophysin and PSD95 in AD, correlating temporal relationship of synaptic dysfunction to onset of cognitive dysfunction in AD patients.

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Education

Johns Hopkins University, Baltimore, MD **May 2016**
- Master of Science & Engineering in Biomedical Engineering

Sir M Visvesvaraya Institute of Technology, India **June 2014**
- Bachelor of Engineering in Biotechnology Engineering
- Gold Medalist for Consistent Distinction

Venkat International Public School, India **May 2009**
- All India Senior School Certificate Examination
- 85.1%, Distinction Holder

Vidya Niketan School, India **May 2007**
- Indian School Certificate Examination, India
- 85.2%, Distinction Holder

Work Experience

Johns Hopkins School of Medicine – Research Assistant Jan 2015 – May 2016
- Assistant in the Dept. of Neurology & Neurosurgery developing and optimizing an extraction protocol for quantitative proteomic studies involved in the oxidative pathways in patients with Alzheimer's disease.
- Executing, optimizing and evaluating different approaches to best protein extraction from human brain samples using lab-prepared buffers and combination of sample disruption equipment.

Johns Hopkins University – Teaching Assistant Sep. 2014 –May 2016
- Grading assignments & tests for Undergraduate students of the BME department (Molecules and Cells, Models and Simulations, Systems Bioengineering-3). Instructor for Biomedical Engineering Practical and Innovation Lab and Systems Bioengineering 2 Lab.

Sir M Visvesvaraya Institute of Technology – Research Assistant
Dec 2013 – July 2014
- Executed comprehensive structure and active site analysis of HSP90, HSP40, HSP70 involved in Alzheimer's disease. Performed computational examination to appreciate intrinsic conformational flexibility of HSP90 to understand protein flexibility and derived a pharmacophore pattern.

Washington University in St. Louis School of Medicine – Intern
July 2012 – Aug 2012

- Carried out spatial temporal control of G protein signaling in single cells using opsins. Used wide field & confocal microscopy to image sensors made of G proteins receptors, effectors. Optimized a protocol for GPCR activation based on G protein subunit fusions with fluorescent proteins

Harvard Medical School - Intern **July 2011 – Aug 2011**

- Assisted a post doctoral student in studying localized and on demand drug delivery from self assembled nanofibrous gels for the prevention and recurrence of brain tumors.
- Designed experimental protocols, collected data and analyzed results for the development of novel biomaterials (hydrogels) as potential therapeutics for brain cancer.

Indian Institute of Science AND Sir MVIT Research Student
Oct 2010 Feb. 2014

- Learnt analytical chemistry techniques to foster better understanding of the workings of a Biotechnology Industry.

University of Waterloo - Information Technology Support Assistant
May- Aug 2010

- Provided help desk support and assisted with software & hardware troubleshooting and installation of network cabling. Built and repaired computer hardware.

Awards and Achievements

- Young Investigator Scholarship Award, Alzheimer's Drug Discovery Foundation, 2016
- Karnataka State Council for Science & Technology – Debate Winner, 2014
  - Placed first amongst 3000 participants state-wide
- Indian Academy of Sciences Fellowship - 2013
  - Was in the top 100 selected from over 10,000 applicants all over India
- International Summer School Scholarship, University of Exeter, 2013
  - Was one of the 145 students selected from all over the world winning a scholarship of £2245
- Best E-Poster "Phantoms in the brain", "Glioblastoma Multiforme", Sir MVIT
- Engineering International Scholarship, University of Waterloo, 2009
- Global Challenge Award, National Science Foundation, 2008
  - #3 spot in Global Rankings from over 600 teams worldwide
- Summer Study Scholarship for Engineering Camp, University of Vermont, 2008

Extracurricular Activities

- Member of Hopkins Cycling **Mar. 2015- Present**
- Member of Hopkins Swimming **Mar. 2015- Present**
- Student Coordinator, Innovation Club, Sir MVIT, India **June 2013 – July 2014**
- Editor of Department Newsletter, Sir MVIT, India **Jan 2013 – July 2014**
- Mentor for Agastya International Foundation, India **Dec. 2012 – May 2014**
  - Trained Government school children in developing Science models
- Member of College Athletic team, Sir MVIT, India **Nov 2011 – June 2014**
- Mentor for the Global Challenge Award **Sept 2008 – May 2009**
  - Led 9 teams from different countries in doing Environmental Engineering Projects such as Solid Waste Management, Environmental Restoration and Project Porch-light

Affiliations

- American Association of Pharmaceutical Scientists (AAPS) **Mar.2015 - Present**
- American Academy of Neurology (AAN) **Mar.2015 - Present**
- Medical Ethics Discussion Panel, Johns Hopkins University **June 2015 - Present**
- Engineers without Borders, Johns Hopkins University **April 2015 - Present**
- Hopkins Graduate Biotech Network, JHU **Feb. 2015 - Present**
- National Alzheimer Advocate Network **July 2015 - Present**

Projects and Assignments

- Finding an EEG Marker for detecting Insomnia in HIV affected patients, Department of Biomedical Engineering, Johns Hopkins University, Fall 2014
- Supply Chain Management, using the Kanban System, December 2009
- Forecasting Coffee Demand ,using time series and regression methods, November 2009

- Work Flow Analysis of Boat Assembly - Determined a learning curve model that described the team's collective boat assembly process. Included an Assembly Line Balancing, October 2009
- The Global Challenge Award - Chosen based on writing a report on the use of biodiesel in the Automobile industry. Involved writing a Technical Description, Market and Industrial Analysis, Business Plan, Political and Financial Feasibility, SWOT Analysis and an Executive Summary, June 2008
- Engineering Sustainability - A project on how to reduce packaging of goods

Conferences and Presentations

- Greater Baltimore Chapter of the Society for Neuroscience (GBSfN) Meeting, University of Maryland, Baltimore, USA, November 2015
  - Presented a poster titled, " A quantitative proteomics study to investigate key proteins involved in oxidative pathways in patients with Alzheimer's Disease"
- Indo-US Translational Neuroscience Symposium 2015, Johns Hopkins School of Medicine, Baltimore, Maryland, USA, February 2015
- Alzheimer's Disease Research Summit 2015, National Institutes of Health, Bethesda, Maryland, USA, February 2015
- National Conference on "Recent advances, career prospects and Entrepreneurial Opportunities in Bioengineering and Biotechnology", March 2014, Sir M Visvesvaraya Institute of Technology, India.
  - Presented a poster titled, "Computational Examination to appreciate intrinsic conformational flexibility of Heat Shock Protein 90 implicated in Alzheimer's disease".
- Have given over 30 presentations on topics such as Intrinsically disordered proteins, Drug Delivery Systems, Isozymes, Bioprocess Equipment Design etc. during the course of my Undergraduate career in Department of Biotechnology
- Indian Institute of Science International Conference on Biomolecular forms and functions, January 2013, Indian Institute of Science, India.
- National Conference on Biopharmaceuticals and Healthcare, November 2011, Sir M Visvesvaraya Institute of Technology, India.
  a. Presented a poster titled, "Drug development for neurodegenerative diseases by Pharmaceutical Companies in India"

Volunteer Experience

- Growing Minds Initiative, Board of Directors, Baltimore **Mar.2015 – Present**
- A Non-Governmental Organization that works on educational needs for Orphaned and Vulnerable Children (OVC) in Tanzania.
- Medical Volunteer Experience
- JHU Visionaries – shadowing medical professionals, conducting patient interviews at the Wilmer Eye Institute

- EEG and Neuroprosthetics Section of the Functional Performance and Device Use Lab at the Food and Drug Administration (FDA) – selected but could not take it up
- Submitted a proposal for the GE Edison Challenge for healthcare innovation in India

➢ Online courses: Fixing Healthcare Delivery, Neurons and Networks, Operations Management, Proteins: Biology's Workforce, Introduction to Computer Science

➢ Model United Nations, Organizing Committee, India **Sep. 2008 – Sep. 2009**

Certifications

1. FDA – Introduction to FDA Human Drug Review and Approval Basics
2. FDA – Centre for Drug Evaluation and Research, Advisory Committee Process Review
3. FDA – Overview of Generic Drugs
4. FDA – Overview of Drug Safety
5. FDA – Role of Office of New Drugs
6. FDA – World of Compliance, Overview
7. Johns Hopkins Medicine – Intermediate Privacy Course for Health Care Providers (Clinicians and Non Clinicians)